

FORM PTO-1390 (REV. 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 265/130
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/890319
INTERNATIONAL APPLICATION NO. PCT/FR00/00105	INTERNATIONAL FILING DATE January 19, 2000	PRIORITY DATE CLAIMED January 27, 1999	
TITLE OF INVENTION METHOD FOR PREPARING MEMBRANE VESICLES			
APPLICANT(S) FOR DO/EO/US Olivier Dhellin; Sebastian Amigorena; Philippe Rameau; and Joel Crouzet			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> has been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input checked="" type="checkbox"/> is attached hereto.</p> <p>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11 to 20 below concern document(s) or information included:</p> <p>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input checked="" type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Return postcard; Intl Search Report; Copy of PCT Request; Original; Annex to Intl Prelimin Exam. Report; Notif Re A La Presen Priorite</p>			

page 1 of 2 of Patents, BOX PCT, Washington, DC 20231
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Lynne Fulmer
Lynne Fulmer

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO.

ATTORNEY'S DOCKET NUMBER

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PCT/FR00/00105

265/130

21. ☐ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1000.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

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\$ 860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS

NUMBER FILED

NUMBER EXTRA

RATE

\$

Total claims 114 - 20 = 94 x \$18.00 \$ 1,692.00

Independent claims 7 - 3 = 4 x \$80.00 \$ 320.00

MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$270.00 \$ 270.00

TOTAL OF ABOVE CALCULATIONS =

\$

☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.

\$

SUBTOTAL =

\$

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).

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TOTAL NATIONAL FEE =

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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Dated: 7/26/2001

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CERTIFICATE

I, Martine NION,

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do hereby declare that I am conversant with the French and English Languages,
and that the attached translation signed by me is, to the best of my knowledge and
belief, a true and correct translation of International Patent Application PCT/FR
00/00105, filed on January 19, 2000.

Dated : June 6, 2001

Signed : M. NION
Martine NION

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METHODS FOR PREPARING MEMBRANE VESICLES

This invention relates to a new method of preparing (particularly isolating and/or purifying) membrane vesicles. The invention also relates to the membrane vesicles prepared in this way and their biological and
5 medical uses, for example.

Membrane vesicles are vesicles, generally less than 100 nm in diameter composed of a lipid bilayer containing a cytosolic fraction. Particular membrane vesicles are more specifically obtained from
10 intracellular compartments through fusion with the plasmic membrane of a cell, resulting in their release in biological fluids or in the supernatant of cells in culture. Such vesicles are generally referred to as exosomes. Exosomes are generally between about 50 and
15 90 nm in diameter, more specifically between about 60 and 80 nm and, advantageously, carry membrane proteins (particularly major histocompatibility complex proteins) which are in the same orientation as in the plasmic membrane of their original cells. In addition,
20 depending on their origin, exosomes comprise membrane proteins such as CD40, CD80 and HSP70 and have no endoplasmic reticulum or Golgi apparatus.

Exosome release has been demonstrated from different cell types in varied physiological contexts.
25 In this way, it has been demonstrated that B lymphocytes release exosomes carrying class II major histocompatibility complex molecules, which play a role in antigenic presentation (Raposo et al., J. Exp. Med.

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183 (1996) 1161). Similarly, it has been demonstrated that dendritic cells produce exosomes (also referred to as dexosomes), with specific structural and functional characteristics and playing a role in immune response mediation, particularly in cytotoxic T lymphocyte stimulation (Zitvogel et al., Nature Medicine 4 (1998) 594). It has also been demonstrated that tumoral cells secrete specific exosomes (also referred to as texosomes) in a regulated manner, carrying tumoral antigens and capable of presenting these antigens or transmitting them to antigen presenting cells (patent application No. WO99/03499). It is also known that mastocyte cells accumulate molecules in intracellular vesicular compartments, which may be secreted under the effect of signals (Smith and Weis, Immunology Today 17 (1996) 60). Therefore, as a general rule, cells appear to emit signals and communicate with each other via membrane vesicles that they release, which may carry antigenic patterns, MHC molecules or any other signal (cytokine, growth factor, etc.) with specific structural and functional characteristics, produced in different physiological situations. Therefore, these vesicles, particularly exosomes, represent a product of particular interest for diagnostic, vaccination or therapeutic applications or to deliver molecules of interest. Therefore, it would be of particular interest to have an effective method which could be used at an industrial scale to prepare membrane vesicles compatible with biological use, particularly pharmacological use.

Conventional methods to prepare membrane vesicles (e.g. exosomes) involve a series of differential centrifugation steps to separate the vesicles from cells or cell debris present in the culture medium. In this way, the documents mentioned above essentially describe the preparation of vesicles with a series of centrifugations at 300 g, 10,000 g and 70,000 g or 100,000 g, the pellet obtained being taken up with a saline solution to constitute a concentrated exosome solution. This preparation may be analysed using conventional biochemical techniques used to evaluate the protein composition of the exosomes. A preferred biochemical technique consists of electrophoresis in a denaturing medium combined with staining of the total proteins or the detection of specific proteins using antibodies according to the Western Blot technique. The exosomes in the final preparation may be detected directly by electron microscopy after fixing the preparation with a 4% glutaraldehyde solution.

According to this process, the purity levels of the exosomes are satisfactory, since such preparations have made it possible to demonstrate the biological activity and the anti-tumoral properties in animal models. However, these prior art centrifugation processes do not enable the fine separation of membrane vesicles (e.g. exosomes) from cell proteins or certain macromolecular components (DNA, RNA) or macromolecular complexes. Therefore, these processes do not exclude the presence of unidentified contaminating biological agents, incompatible with therapeutic use in humans. In

addition, these steps are difficult to extrapolate at an industrial scale, particularly when significant volumes are to be treated, or for autologous (i.e. patient by patient) ex vivo applications, in which the
5 process must generally be applied in a confined system.

The present invention now provides a solution to this problem. The invention describes new processes for the preparation (i.e. isolation and/or purification) of membrane vesicles under conditions compatible with an
10 industrial use and pharmacological applications. In particular, the processes according to the invention may be applied both for individualised autologous exosome preparations and for exosome preparations obtained from established cell lines, for experimental
15 or biological use or prophylactic or therapeutic vaccination purposes, for example.

This invention is more specifically based on the use of chromatography separation methods for preparing membrane vesicles, particularly to separate the
20 membrane vesicles from potential biological contaminants.

More specifically, a first object of this invention resides in a method of preparing membrane vesicles from a biological sample, characterised in that it comprises
25 at least an anion exchange chromatography treatment step of the sample.

Indeed, the applicant has now demonstrated that membrane vesicles, particularly exosomes, could be purified by anion exchange chromatography. In this way,
30 unexpectedly, it is demonstrated in this application

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that exosomes are resolved in a homogeneous peak after anion exchange chromatography. This result is completely unexpected given that exosomes are complex supramolecular objects composed, among other things, of a membrane surrounding an internal volume comprising soluble proteins. In addition, exosomes contain membrane proteins.

Therefore, a preferred object of this invention relates to a method of preparing, particularly of purifying, vesicle membranes, from a biological sample, comprising at least one anion exchange chromatography step.

To apply the invention, a strong or weak, preferably strong, anion exchange may be performed. In addition, in a specific embodiment, the chromatography is performed under pressure. Thus, more specifically, it may consist of high performance liquid chromatography (HPLC).

Different types of supports may be used to perform the anion exchange chromatography. More preferably, these may include cellulose, poly(styrene-divinylbenzene), agarose, dextran, acrylamide, silica, ethylene glycol-methacrylate co-polymer, or mixtures thereof, e.g., agarose-dextran mixtures. To illustrate this, it is possible to mention the different chromatography equipment composed of supports as mentioned above, particularly the following gels : Source, Poros, Sepharose, sephadex, Trisacryl, TSK-gel SW or PW, Superdex, Toyopearl HW and sephacryl, for

example, which are suitable for the application of this invention.

Therefore, in a specific embodiment, this invention relates to a method of preparing membrane vesicles from a biological sample, comprising at least one step during which the biological sample is treated by anion exchange chromatography on a support selected from cellulose, poly(styrene-divinylbenzene), silica, acrylamide, agarose, dextran, ethylene glycol-methacrylate co-polymer, alone or in mixtures, optionally functionalised.

In addition, to improve the chromatographic resolution, within the scope of the invention, it is preferable to use supports in bead form. Ideally, these beads have a homogeneous and calibrated diameter, with a sufficiently high porosity to enable the penetration of the objects under chromatography (i.e. the exosomes). In this way, given the diameter of exosomes (generally between 50 and 100 nm), to apply the invention, it is preferable to use high porosity gels, particularly between 10 nm and 5 μm , more preferably between approximately 20 nm and approximately 2 μm , even more preferably between about 100 nm and about 1 μm .

For the anion exchange chromatography, the support used must be functionalised using a group capable of interacting with an anionic molecule. Generally, this group is composed of an amine which may be ternary or quaternary, which defines a weak or strong anion exchanger, respectively.

Within the scope of this invention, it is particularly advantageous to use a strong anion exchanger. In this way, according to the invention, a chromatography support as described above, functionalised with quaternary amines, is used. Therefore, according to a more specific embodiment of the invention, the anion exchange chromatography is performed on a support functionalised with a quaternary amine. Even more preferably, this support should be selected from poly(styrene-divinylbenzene), acrylamide, agarose, dextran and silica, alone or in mixtures, and functionalised with a quaternary amine.

Examples of supports functionalised with a quaternary amine include the gels Source Q, Mono Q, Q Sepharose, Poros HQ and Poros QE, Fractogel TMAE type gels and Toyopearl Super Q gels.

A particularly preferred support to perform the anion exchange chromatography comprises poly(styrene-divinylbenzene). An example of this type of gel which may be used within the scope of this invention is Source Q gel, particularly Source 15 Q (pharmacia). This support offers the advantage of very large internal pores, thus offering low resistance to the circulation of liquid through the gel, while enabling rapid diffusion of the exosomes to the functional groups, which are particularly important parameters for exosomes given their size.

The biological compounds retained on the column may be eluted in different ways, particularly using the passage of a saline solution gradient of increasing

concentration, e.g. from 0 to 2 M. A sodium chloride solution may particularly be used, in concentrations varying from 0 to 2 M, for example. The different fractions purified in this way are detected by
5 measuring their optical density (OD) at the column outlet using a continuous spectro-photometric reading. As an indication, under the conditions used in the examples, the fractions comprising the membrane vesicles were eluted at an ionic strength comprised
10 between approximately 350 and 700 mM, depending on the type of vesicles.

Different types of columns may be used to perform this chromatographic step, according to requirements and the volumes to be treated. For example, depending
15 on the preparations, it is possible to use a column from approximately 100 μ l up to 10 ml or greater. In this way, the supports available have a capacity which may reach 25 mg of proteins/ml, for example. For this reason, a 100 μ l column has a capacity of approximately
20 2.5 mg of proteins which, given the samples in question, allows the treatment of culture supernatants of approximately 2 l (which, after concentration by a factor of 10 to 20, for example, represent volumes of 100 to 200 ml per preparation). It is understood that
25 higher volumes may also be treated, by increasing the volume of the column, for example.

In addition, to perform this invention, it is also possible to combine the anion exchange chromatography step with a gel permeation chromatography step. In this
30 way, according to a specific embodiment of the

invention, a gel permeation chromatography step is added to the anion exchange step, either before or after the anion exchange chromatography step. Preferably, in this embodiment, the permeation
5 chromatography step takes place after the anion exchange step. In addition, in a specific variant, the anion exchange chromatography step is replaced by the gel permeation chromatography step. The present application demonstrates that membrane vesicles may
10 also be purified using gel permeation liquid chromatography, particularly when this step is combined with an anion exchange chromatography or other treatment steps of the biological sample, as described in detail below.

15 To perform the gel permeation chromatography step, a support selected from silica, acrylamide, agarose, dextran, ethylene glycol-methacrylate co-polymer or mixtures thereof, e.g., agarose-dextran mixtures, are preferably used. As an illustration, for gel permeation
20 chromatography, a support such as Superdex 200HR (Pharmacia), TSK G6000 (TosoHaas) or Sephacryl S (Pharmacia) is preferably used.

The process according to the invention may be applied to different biological samples. In particular,
25 these may consist of a biological fluid from a subject (bone marrow, peripheral blood, etc.), a culture supernatant, a cell lysate, a pre-purified solution or any other composition comprising membrane vesicles.

In this respect, in a specific embodiment of the invention, the biological sample is a culture supernatant of membrane vesicle-producing cells.

In addition, according to a preferred embodiment of the invention, the biological sample is treated, prior to the chromatography step, to be enriched with membrane vesicles (enrichment stage). In this way, in a specific embodiment, this invention relates to a method of preparing membrane vesicles from a biological sample, characterised in that it comprises at least:

b) an enrichment step, to prepare a sample enriched with membrane vesicles, and

c) a step during which the sample is treated by anion exchange chromatography and/or gel permeation chromatography.

According to a preferred embodiment, the biological sample is a culture supernatant treated so as to be enriched with membrane vesicles. In particular, the biological sample may be composed of a pre-purified solution obtained from a culture supernatant of a population of membrane vesicle-producing cells or from a biological fluid, by treatments such as centrifugation, clarification, ultrafiltration, nanofiltration and/or affinity chromatography, particularly with clarification and/or ultrafiltration and/or affinity chromatography.

Therefore, a preferred method of preparing membrane vesicles according to this invention more particularly comprises the following steps:

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The cells and/or cell debris may also be eliminated by filtration of the sample, possibly combined with the centrifugation described above. The filtration may particularly be performed with successive filtrations using filters with a decreasing porosity. For this purpose, filters with a porosity above 0.2 μm , e.g. between 0.2 and 10 μm , are preferentially used. It is particularly possible to use a succession of filters with a porosity of 10 μm , 1 μm , 0.5 μm followed by 0.22 μm .

A concentration step may also be performed, in order to reduce the volumes of sample to be treated during the chromatography stages. In this way, the concentration may be obtained by centrifugation of the sample at high speeds, e.g. between 10,000 and 100,000 g, to cause the sedimentation of the membrane vesicles. This may consist of a series of differential centrifugations, with the last centrifugation performed at approximately 70,000 g. The membrane vesicles in the pellet obtained may be taken up with a smaller volume and in a suitable buffer for the subsequent steps of the process.

The concentration step may also be performed by ultrafiltration. In fact, this ultrafiltration allows both to concentrate the supernatant and perform an initial purification of the vesicles. According to a preferred embodiment, the biological sample (e.g., the supernatant) is subjected to an ultrafiltration, preferably a tangential ultrafiltration. Tangential ultrafiltration consists of concentrating and

fractionating a solution between two compartments (filtrate and retentate), separated by membranes of determined cut-off thresholds. The separation is carried out by applying a flow in the retentate compartment and a transmembrane pressure between this compartment and the filtrate compartment. Different systems may be used to perform the ultrafiltration, such as spiral membranes (Millipore, Amicon), flat membranes or hollow fibres (Amicon, Millipore, Sartorius, Pall, GF, Sepracor). Within the scope of the invention, the use of membranes with a cut-off threshold below 1000 kDa, preferably between 300 kDa and 1000 kDa, or even more preferably between 300 kDa and 500 kDa, is advantageous.

The affinity chromatography step can be performed in various ways, using different chromatographic support and material. It is advantageously a non-specific affinity chromatography, aimed at retaining (i.e., binding) certain contaminants present within the solution, without retaining the objects of interest (i.e., the exosomes). It is therefore a negative selection. Preferably, an affinity chromatography on a dye is used, allowing the elimination (i.e., the retention) of contaminants such as proteins and enzymes, for instance albumin, kinases, deshydrogenases, clotting factors, interferons, lipoproteins, or also co-factors, etc. More preferably, the support used for this chromatography step is a support as used for the ion exchange chromatography, functionalised with a dye. As specific example, the dye

may be selected from Blue Sepharose (Pharmacia), Yellow 86, Green 5 and brown 10 (Sigma). The support is more preferably agarose. It should be understood that any other support and/or dye or reactive group allowing the retention (binding) of contaminants from the treated biological sample can be used in the instant invention.

In a specific embodiment of the invention, the biological sample is obtained by subjecting a membrane vesicle-producing cell culture supernatant to at least one filtration stage.

In another specific embodiment of the invention, the biological sample is obtained by subjecting a membrane vesicle-producing cell culture supernatant to at least one centrifugation step.

In a preferred embodiment of the invention, the biological sample is obtained by subjecting a membrane vesicle-producing cell culture supernatant to at least one ultrafiltration step.

In another preferred embodiment of the invention, the biological sample is obtained by subjecting a membrane vesicle-producing cell culture supernatant to at least one affinity chromatography step.

A more specific preferred membrane vesicle preparation process within the scope of this invention comprises the following steps:

- a) the culture of a population of membrane vesicle (e.g. exosome) producing cells under conditions enabling the release of vesicles,

b) the treatment of the culture supernatant with at least one ultrafiltration or affinity chromatography step, to produce a biological sample enriched with membrane vesicles (e.g. with exosomes), and

5 c) an anion exchange chromatography and/or gel permeation chromatography treatment of the biological sample.

In a preferred embodiment, step b) above comprises a filtration of the culture supernatant, followed by an
10 ultrafiltration, preferably tangential.

In another preferred embodiment, step b) above comprises a clarification of the culture supernatant, followed by an affinity chromatography on dye, preferably on blue sepharose.

15

In addition, after step c), the material harvested may, if applicable, be subjected to one or more additional treatment and/or filtration stages d), particularly for sterilisation purposes. For this
20 filtration treatment stage, filters with a diameter less than or equal to $0.3\ \mu\text{m}$ are preferentially used, or even more preferentially, less than or equal to $0.25\ \mu\text{m}$. Such filters have a diameter of $0.22\ \mu\text{m}$, for example.

25 After step d), the material obtained is, for example, distributed into suitable devices such as bottles, tubes, bags, syringes, etc., in a suitable storage medium. The purified vesicles obtained in this way may be stored cold, frozen or used
30 extemporaneously.

Therefore, a specific preparation process within the scope of the invention comprises at least the following steps:

5 c) an anion exchange chromatography and/or gel permeation chromatography treatment of the biological sample, and

d) a filtration step, particularly sterilising filtration, of the material harvested after stage c).

10 In a first variant, the process according to the invention comprises:

c) an anion exchange chromatography treatment of the biological sample, and

15 d) a filtration step, particularly sterilising filtration, on the material harvested after step c).

In another variant, the process according to the invention comprises:

20 c) a gel permeation chromatography treatment of the biological sample, and

d) a filtration step, particularly sterilising filtration, on the material harvested after step c).

25 According to a third variant, the process according to the invention comprises:

c) an anionic exchange treatment of the biological sample followed or preceded by gel permeation chromatography, and

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d) a filtration step, particularly sterilising filtration, on the material harvested after step c).

The results given in the examples demonstrate that the injection of an exosome preparation into the chromatography column allows to obtain symmetric absorption peaks, which are perfectly resolved (Fig. No. 1). The different fractions isolated in this way may be analysed using conventional protein electrophoresis techniques in denaturing gel followed by Coomassie blue staining techniques or specific protein detection techniques using antibodies. It is thus possible to demonstrate, for texosomes, that the peak eluted in anion exchange chromatography with a 400 mM saline solution has an identical protein profile to that of an exosome preparation prepared using conventional methods (Fig. No. 2). In fact, this allows to characterise the peaks eluted at lower or higher saline concentrations as related to distinct, biological contaminants. In another experiment, membrane vesicles produced from dendritic cells (dexosomes) or certain texosomes are eluted at a ionic strength comprised between about 500 and 700 mM, and mastocyte-derived vesicles at about 350 mM.

Furthermore, the results given in the examples also demonstrate that the method according to the invention allows the detection of the possible contamination of the preparation by major proteins of the culture medium such as bovine serum albumin. Indeed, the chromatography of a standard bovine serum albumin

solution under the above conditions demonstrates a peak eluted at a distinct saline concentration from that of the exosomes (Fig. No. 3).

Therefore, the process according to the invention
5 allows to (i) purify membrane vesicles under quality and quantity conditions compatible with pharmacological use, and (ii) detect the existence of distinct biological contaminants in the biological sample treated.

10 The process according to the invention may be applied to the preparation of membrane vesicles of varied origins. In particular, these vesicles may consist of exosomes produced by antigen presenting cells or by tumour cells, for instance. In addition,
15 they may consist of primary cells, for example in culture, or of established lines; e.g., immortalised membrane vesicle-producing lines. They may consist of cells of Mammalian origin, particularly of murine or human origin.

20 In a specific embodiment of the invention, the membrane vesicles are vesicles produced by antigen presenting cells, particularly dendritic cells, B lymphocytes, macrophages and mastocytes, optionally after sensitisation of these cells to one or more
25 selected antigens. A particularly preferred application of this invention is based on the preparation of membrane vesicles produced by dendritic cells. These may be human or animal dendritic cells, particularly of human or murine origin. These cells may be primary
30 cells, harvested from a subject's biological fluids or

produced ex vivo from precursor cells, or also from established line cells, immortalised with an oncogene, for example (EP 701 604).

A specific object of this invention resides in a method of preparing membrane vesicles (dexosome), characterised in that it comprises the following steps :

- a) obtaining a population of dendritic cells,
- b) culturing the dendritic cells under conditions enabling the production of membrane vesicles (dexosomes), and
- c) purifying the membrane vesicles (dexosomes) using a process comprising at least an anion exchange chromatography treatment as described above.

A more preferred embodiment resides in a method of preparing dexosomes, comprising the following steps:

- a) obtaining a population of dendritic cells,
- b) culturing the dendritic cells under conditions enabling the production of dexosomes,
- c) treating the culture supernatant to produce a biological sample enriched with dexosomes, particularly with an ultrafiltration or an affinity chromatography step, and
- d) purifying the dexosomes using a process comprising at least an anion exchange and/or gel permeation chromatography stage, under the conditions described above.

More specifically, for the performance of this variant of the invention, the dendritic cells are

preferentially obtained from a biological sample from a subject, e.g. bone marrow or peripheral blood.

In this respect, dendritic cell production techniques have been described in the prior art and may
5 be used by the skilled artisan (see particularly the techniques described in the application WO99/03499, incorporated to this application by reference). The dendritic cells may thus be prepared from immune system stem cells, monocyte precursors or be isolated directly
10 in a differentiated state (Review by Hart, Blood 90 (1997) 3245).

A preferred methodology within the scope of this invention is based on the production of dendritic cells from monocyte precursors or bone marrow. More
15 specifically, within the scope of this invention, it is preferred to use dendritic cells obtained by treating monocyte precursors (contained in blood or marrow) in the presence of a GM-CSF+IL-4 or GM-CSF+IL-13 combination.

20 In addition, for the performance of this invention, it is particularly advantageous to use a population of dendritic cells comprising immature dendritic cells. Advantageously, a population of dendritic cells composed mainly (i.e. at least 60%, preferably 70%) of
25 immature dendritic cells is used.

Therefore, the dendritic cell preparation step may advantageously comprise the preparation of a dendritic cell population comprising immature dendritic cells, particularly of human origin, especially from monocyte
30 precursors, more specifically by treatment with a

combination of cytokines such as GM-CSF+IL-4 or GM-CSF+IL-13.

In addition, within the scope of this invention, it is also possible to use immortalised dendritic cell populations. These may consist of immortalised dendritic cell lines (e.g. D1 line or any other line produced by introducing the myc oncogene in the dendritic cells, for example). They may also consist of dendritic cells prepared and immortalised in vitro. The interest of immortalised dendritic cells lies in the constitution of banks of cells sensitised to given antigen groups, which may be used industrially to prepare dexosomes compatible for administration to entire families of patients.

To produce the membrane vesicles (dexosomes), the dendritic cells may be simply cultured under conventional conditions known to those skilled in the field. However, it is preferred to culture these cells under conditions stimulating the production of dexosomes, particularly in the presence of factors capable of stimulating dexosome production, particularly a cytokine such as gamma interferon, interleukin 10 or interleukin 12 (e.g. see application WO99/03499). In a preferred embodiment of the process according to the invention, the dendritic cells are cultured, during step b) described above, under conditions stimulating membrane vesicle production.

Moreover, in a specific variant, the dendritic cells are sensitised to an antigen prior to membrane vesicle production. This embodiment allows the

concentration of dendritic cells with specific antigen(s), so as to produce dexosomes with a given immunogenicity. The sensitisation may be performed using different well-known techniques, comprising for example placing the cells in contact with antigenic peptides, antigens, protein complexes, cells or membranes of cells expressing antigens, apoptotic bodies, membrane vesicles, liposomes, tumoral RNA or any nucleic acid coding for one or more antigens, antigenic determinants or epitopes (possibly carried by a viral or non-viral vector), etc. (e.g. see application WO99/03499). In a preferred method, the sensitisation is performed by incubation with peptides, antigens, RNA or nucleic acids. It is understood that this application is not limited to dendritic cell sensitisation or production techniques.

Another particularly advantageous embodiment of this invention is based on the preparation of membrane vesicles produced by tumoral cells, especially of human origin. In particular, they may consist of any cell from a solid or liquid tumour and cells transformed or immortalised in vitro. Solid, haematopoietic or ascitic tumours may be mentioned more preferentially.

A specific embodiment of this invention also resides in the preparation of membrane vesicles comprising one or more heterologous molecules, particularly recombinant molecules. In this way, it is possible, for example, to genetically modify membrane vesicle-producing cells (e.g., mastocyte lines), to make them express, in or at the surface of the vesicles

they produce, molecules of interest (PCT/FR99/02691). This invention may also be used to purify such modified vesicles.

More generally, the invention may be applied to the preparation of membrane vesicles produced by any cell type, particularly exosome type vesicles, preferably with a diameter less than approximately 100 nm. These may consist of macrophage cells, mastocytes, reticulocytes, etc. In the experimental section, exosome preparations from (i) cell lines such as murine tumoral cell lines (TS/A), dendritic cell lines (D1), mastocyte cell line (RBL) and (ii) human monocyte-derived dendritic cells have been used. The results obtained can be transposed directly to other primary cultures such as human tumoral cells, dendritic cells, B lymphocytes, etc., cultivable under industrially acceptable conditions. The process according to the invention may thus be used in the purification of exosomes to be used in human treatment.

Depending on their origin, the membrane vesicles obtained in this way represent tools for the study of cancer or immune system regulation and for molecule transfer, antibody production, labelling, diagnosis, bank constitution, vaccine or medicinal product ingredients, etc.

In addition, the process according to the invention may also be used as a method for quality control on the possible presence of contaminants (particularly contaminating proteins) in the culture medium or in membrane vesicle preparations.

Therefore, in this respect, this invention may be applied both in a vesicle membrane preparation method and in an analytical system used to test the quality of a membrane vesicle preparation, irrespective of the preparation method.

Therefore, this invention also relates to a process to test for the presence of contaminants, particularly of protein or nucleic origin, in a membrane vesicle, particularly exosome, preparation, comprising
10 subjecting a fraction of said preparation to at least an anion exchange chromatography and the detection of the presence of contaminants.

The invention also relates to, as a general rule, the use of anion exchange chromatography, particularly
15 high performance liquid chromatography, for the preparation or purification of membrane vesicles. It also relates to the use of affinity chromatography for the preparation or purification of membrane vesicles.

The invention also relates to the membrane vesicles
20 prepared using the process according to the invention, and any composition comprising such vesicles.

This invention will be described more completely using the following examples which must be considered as illustrative and not restrictive.

25

Legend to the figures

Figure No. 1: Elution profile after anion exchange chromatography of a sample of exosomes prepared by differential centrifugation.

Figure No. 2: Protein profile analysis, by electrophoresis in SDS PAGE followed by Coomassie blue staining, of the different elution fractions of an exosome preparation.

5 Figure No. 3: Elution profile after anion exchange chromatography of a standard bovine serum albumin.

Figure No. 4: Tangential ultrafiltration treatment diagram of a biological sample comprising membrane vesicles.

10 Figure No. 5: General profile of the Blue Sepharose 6 fast flow step, following 600 and 10 000 g centrifugations of a dexosome supernatant.

Figure No. 6: Western blot against MHCI molecules of the exosomes, in the non-adsorbed fraction and the eluate of the Blue Sepharose 6 fast flow step.

15 Figure No. 7: General profile of the Source Q15 step following a Blue Sepharose 6 fast flow step

Figure No. 8: Detail of the elution gradient on the Source Q15 following a Blue Sepharose 6 fast flow step

20 Figure No. 9: Purification profile of exosomes produced by RBL DR+ cell line (eq. 53 μ g proteins) and purified on Source Q15 column after Dnase and Rnase treatment.

25 Figure No. 10: Separation of exosomes by a discontinued NaCl gradient on a Source Q15 support.

Figure No. 11: Western blot against human MHCI molecules of each peak separated by HPLC and ultracentrifuged. Positive control: human dendritic

30 cells expressing CMHII molecules. Negative control:

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ultracentrifuged medium (background signal). MW: molecular weight; NA: Non-Adsorbed fraction.

General cell culture and molecular biology techniques

5

1) Cell cultures

The TS/A cell line is a murine cell line established from a spontaneous mammary carcinoma. This line is cultured at 37°C in the presence of 5% CO₂ in
10 RPMI medium in the presence of 10% foetal calf serum (Dominique Dutcher).

The dendritic cell lines are kept in an IMDM medium containing 10% inactivated foetal bovine serum, 2 mM of L-glutamine, 50 µM of 2-βME, 100 UI/ml of penicillin
15 and 100 µg/ml of streptomycin.

2) SDS PAGE protein analysis

20 µl of sample is diluted in Laemmli buffer (Nature 227 (1970) p.680-685) and then subjected to thermal denaturation at 95°C for 10 min and then loaded
20 on 10% acrylamide gels (Novex 1 mm x 10 wells). After migration, the gels are stained with Coomassie blue.

Examples

25 1) ANION EXCHANGE CHROMATOGRAPHY OF AN EXOSOME PREPARATION PRODUCED BY TUMORAL CELLS (TS/A LINE): SDS PAGE PROTEIN PROFILE ANALYSIS OF THE DIFFERENT ELUTED FRACTIONS.

This example illustrates how an anion exchange chromatography treatment can be used to separate impurities from an exosome preparation.

1.1. Protocol:

5 In this experiment, the starting material is made up of a concentrate of exosomes prepared by differential centrifugation from a TS/A cell culture supernatant, used to separate the exosomes from the cells or cell debris present in the culture medium. The
10 first centrifugation is performed at a low speed (300 g for 5 min) in order to cause the sedimentation of the cells in suspension present in the culture supernatant. Two other centrifugations (1200 g for 20 min followed by 10,000 g for 30 min) are carried out to cause the
15 pelleting of the cell debris. The supernatant clarified in this way is then subjected to ultracentrifugation at a high speed of 70,000 g for 1 hour used to cause the sedimentation of the exosomes. This preparation is then washed in a large volume of saline solution to be
20 recentrifuged under the above conditions. The pellet is then taken up with a volume of approximately 100 μ l of saline solution and forms a concentrated exosome solution. The quantity of proteins is measured using the Bradford technique (Biorad, Ivry, France). This
25 concentrate has a total protein content between 500 and 1000 μ g/ml.

40 μ g of this exosome preparation diluted in 500 μ l of pH 8 50 mM HCl/Tris buffer is injected onto a column containing Source Q 15 gel (Pharmacia) stabilised in a
30 pH 8 50 mM HCl/Tris buffer. After rinsing, the adsorbed

species are eluted on 30 volumes of column with a linear 0 to 500 mM NaCl gradient, followed by a 2 M NaCl solution. The elution fractions are analysed by spectrophotometry at 260 and 280 nm. The elution
5 fractions are grouped into 5 major fractions (F1 to F5), to analyse their respective protein profile. The proteins of each fraction are precipitated with 1/10 volume of a 100% trichloroacetic acid solution and then rinsed with an acetone solution. The protein pellets
10 are taken up with 20 μ l of Laemmli solution and are deposited on an SDS PAGE acrylamide gel which is then stained with Coomassie blue.

1.2. Results:

The elution profiles at 260 and 280 nm are shown on
15 figure 1. The profiles show the presence of 3 distinct symmetric peaks, eluted at the respective saline concentrations of 105 mM, 400 mM and 2 M NaCl.

The protein profile analysis of the fractions corresponding to the different peaks shows that the
20 peak eluted at 400 mM NaCl has an identical protein profile to that of a conventional exosome preparation prepared by centrifugation (Figure No. 2). The ratio between the absorptions at 260 and 280 nm measured at a value of 0.8 is compatible with that described for
25 proteins.

However, the peak eluted (fraction F2) at 105 mM NaCl shows a different protein profile, revealing only two distinct bands. The absorbance measurements at 260 and 280 nm give a ratio of 1.6 suggesting the presence
30 of a combination of nucleic acids and proteins.

The fraction F5 eluted at 2 M NaCl corresponds to the biological compounds strongly linked to the column.

It is therefore possible to separate exosome from impurities with an anion exchange step.

5

2) ANION EXCHANGE CHROMATOGRAPHY OF A STANDARD BOVINE SERUM ALBUMIN SOLUTION

This anion exchange chromatography technique can also be used to evaluate the degree of contamination of an exosome preparation by the proteins present in the culture medium. In this way, by performing chromatography on 10 μ g of a bovine serum albumin solution, corresponding to the majority protein in the culture medium, we demonstrate that it is eluted at 205 mM NaCl in the form of a narrow peak distinguishable from the peaks described above for exosomes.

3) ULTRAFILTRATION TREATMENT OF A CULTURE SUPERNATANT CONTAINING EXOSOMES.

This example demonstrates that it is possible to concentrate exosomes by ultrafiltration. In addition, the exosomal preparation is low in contaminant proteins such as BSA.

Protocol: (Figure No. 4)

150 ml of a cell culture supernatant obtained after centrifugation of TSA cells at 300 g is subjected to a filtration on a 0.22 μ m porosity filter (Millipore). The filtrate is diluted by half in PBS. The resulting 300 ml is filtered tangentially with a filtration

cassette (10 cm² of membrane) with a cut-off threshold of 300,000 D (Sartorius).

Results:

7 ml of retentate are obtained after 1 hour of
5 filtration. The retentate was subjected to an
ultracentrifugation (79,000) to cause the pelleting of
and analyse the exosomes. An SDS-PAGE analysis followed
by Coomassie blue staining revealed that the sample
10 contained significantly less BSA than the solution
which was ultrafiltered. In addition, specific protein
bands of the exosomes produced from TSA are observed.

Therefore, ultrafiltration may be used in an
exosome purification process in order to separate the
exosomes from contaminating proteins.

15

4. PURIFICATION BY HPLC OF HUMAN EXOSOMES, PRODUCED BY
HUMAN MONOCYTE-DERIVED DENDRITIC CELLS (MDDC)

4.1) Materials and Methods

20

. Buffers and stock solutions

0.22 µm filtered stock solutions are used, except
for the water and soda solution. The first buffer is a
100 mM (Sigma, 99% purity) Bis-Tris-Propane (BTP)
25 solution, buffered to pH 6; this buffer is connected to
channel A of the chromatograph (BioCad Sprint, Perkin-
Elmer). The second buffer is a 100 mM Bis-Tris-Propane
solution, buffered to pH 9; this buffer is connected to
channel B of the chromatograph. The water is produced
30 on resin with a Milli-Q system at a resistance of 18

MΩcm. The water is connected to channel C. A 3 M sodium chloride stock solution (NaCl, Prolabo, 99.5% purity) is connected to channel D. A 0.1 M soda solution (NaOH, Prolabo, 98% purity minimum) is connected to channel F. Channel E is used to load the culture supernatants onto the columns.

All the buffers are produced from water produced by the Milli-Q system and are not degassed.

. Columns

10 Blue Sepharose 6 fast flow (Pharmacia):

The first step is performed with a Blue Sepharose 6 fast flow (Pharmacia) column. The matrix is agarose linked with Blue Sepharose 6 fast flow Blue 3G (7%). The particle size is between 45 and 165 μm. The maximum linear flow rate is 750 cm/hour. The gel is stable at pH values between 4 and 12; at the extreme pH values, 4 and 12, the gel may be damaged, inducing a decrease in the fixing capacity (segregation of the Blue Sepharose 6 fast flow) and an increase in pressure (fine formation).

Blue Sepharose 6 fast flow is specific for compounds such as albumin, kinases, dehydrogenases and other enzymes containing cofactors such as NAD⁺, clotting factors, interferons and lipoproteins.

25 The theoretical fixing capacity of Blue Sepharose 6 fast flow is approximately 15 to 20 mg of serum albumin per ml of gel.

The column volume used is approximately 5.5 ml of gel (C10/10, Pharmacia), or a theoretical capacity of 30 80 to 110 mg of serum albumin. The flow rate used is 2

ml/min (150 cm/hour) on the BioCad Sprint and 3.5 ml/min (260 cm/hour) with a Pharmacia detection system. The pressure does not exceed 2.5 bar and is essentially due to the OD measurement cells.

5 Source 15Q:

The second step is performed with a Source 15Q (Pharmacia) column, a strong anion exchanger. The matrix is polystyrene cross-linked with divinyl benzene. The bead size is 15 μ m and homogeneous. The
10 beads are passed through a system of pores with a size varying from 20 to 1000 nm. These gels are very resistant to pressure and withstand high linear flow rates (1800 cm/hour and over), while retaining a satisfactory resolution and capacity. This is made
15 possible by the homogeneity of the beads and their porosity which increases the access of the molecules to the functional groups.

The gel is stable at pH values between 2 and 12, beyond which the gel is liable to undergo significant
20 damage, reducing its capacities.

The theoretical fixing capacity of this exchanger is approximately 25 mg of proteins per ml of gel. A 0.8 ml column (PEEK column 4.6 mm ID/50 mm L, Perkin-Elmer) is used, giving a maximum capacity of 20 mg of
25 proteins. The actual capacity, ensuring a good resolution, is approximately 10% of this maximum capacity, or 2 mg of proteins. The flow rate used is 5 ml/min (1880 cm/hour) and allows quick separations. The column is packed with the Poros selfPack system at 15
30 ml/min at 150 bar.

. Chromatograph (BioCad Sprint)

The BioCad is an HPLC (High Performance Liquid Chromatography) system which makes it possible to work at high pressures (maximum bar) and at flow rates ranging from 0.2 to 60 ml/min. Up to 6 buffers may be connected (the 6 channels are currently used) and the system may be treated with 0.1 M soda (pH 12) to depyrogenate the tubing and column.

The separations may be performed at ambient temperature or at 4°C. The samples are loaded either via one of the accessible channels or via injection loops for small volumes (100 µl to 5 ml). The detection system uses a double-channel UV cell: 190 to 450 nm for UV and 366 to 700 nm for visible. Conventionally, a 254 nm detection is used for nucleic acids and a 280 nm detection for proteins (it is the amino acids with a benzene cycle such as tyrosins, tryptophans and phenylamines that absorb). The system is entirely computerised (software developed by Perspective biosystem). For each separation, all the parameters (pressure, flow rate, conductivity, optical density, pH, etc.) can be monitored.

Finally, the separated sample is either recovered in a 50 ml Falcon tube or collected in siliconed Eppendorf tubes (Advantec SF-2120 collector) to minimise non-specific interactions.

. Exosome production from dendritic cells

Firstly, the dendritic cells are obtained from peripheral blood monocyte precursors. The isolated monocytes are cultured in the presence of a combination

of GM-CSF and IL-13 or IL-4 (see the techniques described in the application WO99/03499). For the production of exosomes, it is preferable to use a population of immature dendritic cells.

5

4.2) Blue Sepharose 6 fast flow stage

The culture supernatants are centrifuged twice at 600 g and once at 10,000 g before being loaded onto the
10 Blue Sepharose 6 fast flow column.

A 5.5 ml column is used, the flow rate is 2 ml/min (linear flow rate 150 cm/hour, contact time 2.7 min) and the pressure is approximately 2.5 bar (fig 5). The column is equilibrated in 12 mM BTP buffer (150 mM
15 NaCl, pH 7). After equilibration, the supernatant is loaded onto the column which is then washed with the same equilibrating buffer until the O.D. drops. The fixed proteins are eluted in 12 mM BTP buffer (1.5 mM NaCl, pH 7) (in 3 to 4 column volumes). The column is
20 regenerated by passing water (2 to 3 column volumes), followed by 2 volumes of 0.1 M soda (pH 12). Finally, the column is restabilised in 12 mM BTP (150 mM NaCl, pH 7).

25 Quantitative and qualitative aspect of the Blue Sepharose 6 fast flow stage

As described above, the Blue Sepharose 6 fast flow stage is specific for proteins such as albumin which is
30 a major contaminant of the culture supernatant. The

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protein concentration of each fraction of the Blue Sepharose 6 fast flow stage is measured using a Biorad technique (OD measurement at 600 nm). The results are given in table 1.

5

Table 1. Protein concentration in each fraction of the Blue Sepharose 6 fast flow stage.

	supernatant	Non-adsorbed	Elution peak	Regeneration peak
Protein concentration (mg/ml)	3.69	0.18	3.32	1.48
Volume (ml)	25	37.5	20	20
total quantity of proteins (mg)	92.2	6.7	66.4	29.6
Yields (%)	100	7.3	72	32.1

10 Most of the proteins are detected either in the eluate (72%) or in the regeneration (32%). The non-adsorbed fraction only represents 7 to 10% of the total proteins loaded onto the Blue sepharose 6 fast flow column. The stage is specific for the major
 15 contaminants of the supernatant. To check this specificity of Blue sepharose 6 fast flow, each fraction is deposited on an SDS-PAGE gel under reducing conditions and stained with silver nitrate. The overload of the column (50 ml supernatant loaded onto a
 20 5.5 ml Blue sepharose 6 fast flow column) was used to

calculate the maximum quantity of proteins of the culture supernatant that it is possible to load onto the column per ml of gel (table 2). In this case, the percentage of the non-adsorbed fraction increases from 7 to over 30% of the quantity of protein loaded. This value is between 16 and 18 mg of proteins per ml of Blue sepharose 6 fast flow gel. In conclusion, 1 ml of Blue sepharose 6 fast flow gel allows the purification of approximately 5 to 6 ml of culture supernatant (2.5% HSA AIMV). This value is very important for the scale-up study since it determines the size of the column to use and, consequently, the cost of this stage.

Table 2. Overload study of the Blue sepharose 6 fast flow stationary phase in the first stage.

	Supernatant	Non-adsorbed	Elution peak	Regeneration peak
Protein concentration (mg/ml)	3.02	0.87	3	1.56
Volume (ml)	50	60	20	20
total quantity of proteins (mg)	151	52.2	60	31.2
Yields (%)	100	34.6	39.7	20.6

As expected, the major contaminant of the culture supernatants, after the 600 and 10,000 g centrifugations, is albumin. After fixing on the Blue

sepharose 6 fast flow, this contaminant is detected in the eluate and regeneration fractions. In addition to this contaminant, many other low and high molecular weight contaminants are detected.

5 The Blue sepharose 6 fast flow stage enables the elimination of approximately 90 to 95% of the culture supernatant contaminants. The exosomes are found in the non-adsorbed fraction of the Blue sepharose 6 fast flow (fig. 6).

10

4.3) Anion exchanger step: source 15Q (Pharmacia).

A 0.8 ml source 15Q column is used with a 5 ml/min flow rate (linear flow rate 1880 cm/hour, contact time 15 0.1 min) with a pressure of approximately 50 bar. The non-adsorbed fraction of the Blue sepharose 6 fast flow is loaded directly onto the source 15Q (fig. 7) with no modifications in the saline (NaCl) concentration or the pH.

20 After the binding stage, the column is washed in 12 mM BTP buffer (280 mM NaCl) at pH 7 (35 column volumes) until the OD drops to values close to 0. A second washing stage is performed at a 150 mM NaCl saline concentration (10 column volumes) to reinforce the 25 interactions of the exosomes with the support (stationary phase).

A first gradient from 150 to 420 mM NaCl in 7 column volumes (fig. 8) is carried out and the gradient is then stopped for 9 column volumes. The second 30 gradient starts from 420 mM NaCl to 1 M NaCl in 25

column volumes. The exosomes are eluted in the second gradient in two peaks at 550 and 700 mM NaCl (fig. 8). The column is regenerated by passing 10 column volumes of water followed by 10 column volumes of 0.1 M soda (pH 12) and, finally, by passing 10 column volumes of 3 M NaCl. The column is then equilibrated in 12 mM BTP buffer (150 mM NaCl, pH 7).

Quantitative and qualitative aspects of the source 15Q stage.

As illustrated in figure 6, most of the proteins of the non-adsorbed Blue sepharose 6 fast flow fraction are not retained on the column. The protein concentrations were measured using a Biorad test (OD absorption at 600 nm) and are summarised in table 3.

Table 3. Quantitative aspects of the Source 15Q stage after a Blue sepharose 6 fast flow stage.

	Start (non-adsorbed Blue sepharose 6 fast flow)	Non-adsorbed source 15Q	Eluate fractions 4 to 6	Eluate fraction s 19 to 23
Proteins (mg/ml)	0.17	0.14	0.01	0.02
Volume (ml)	85	98	3	5
Total proteins (mg)	14.45	13.72	0.03	0.10

Yields (%)	100	94.9	0.2	0.8
------------	-----	------	-----	-----

95% of the proteins loaded on the Source 15Q column are not retained. The pooled fractions 4 to 6 and the pooled fractions 19 to 23 represent approximately 1.5%.
 5 The regeneration was not assayed. The yield is very close to 100%: all the loaded proteins and vesicles are eluted from the column. In terms of the column's protein concentration, Source 15Q is capable of binding approximately 25 mg of proteins (manufacturer's data),
 10 corresponding to 20 mg of proteins for 0.8 ml columns. It is clear that these values are very far from the column's maximum and the 5 to 10% for a good resolution, since they range between 1 and 2 mg or proteins. Under these conditions, with a 0.8 ml Source
 15 15Q column, it is possible to purify between 200 and 400 ml of culture supernatant.

Each elution peak is ultracentrifuged (100,000 g for 1 hour) and pooled for electron microscope observation. The first peak (eluted between 150 and 420
 20 mM NaCl) essentially contains proteins, cell debris and some vesicles labelled with an anti-MHC II antibody.

The second peak (eluted at 550 mM NaCl), processed in the same way, shows a lower background noise and a much higher number of vesicles labelled with an anti-
 25 MHC II antibody; the size of the vesicles is also heterogeneous.

The third peak (eluted at 700 mM NaCl) does not show any background noise, the vesicles are practically

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all labelled with an anti-MHC II antibody and are much more homogeneous in size.

These two fractions (peak 2 and 3) are to be compared to those obtained using the conventional ultracentrifugation purification process. In this case, a significant background noise and a considerable heterogeneity of the vesicles compared to the two chromatography peaks are observed. In addition, there appears to be a separation between the debris and the exosomes purified by HPLC which is not the case with ultracentrifugation.

4.4) Conclusions

In two chromatography stages, the first involving a negative selection (Blue sepharose 6 fast flow stage) of exosomes and the second involving a positive selection and a selective elution of exosomes (Source 15Q stage), approximately between 99 and 99.5% of the proteins in the culture supernatant are eliminated, while retaining the exosomes. The process preserves the integrity of the exosomes in electron microscopy. Therefore, this process is specific for exosome purification.

As an example, other columns retaining serum proteins may be used. In addition, cationic macroporous columns may be used instead of Source 15Q.

5) PURIFICATION BY HPLC OF EXOSOMES PRODUCED FROM THE RBL (RAT BASOPHILIC LEUKEMIA) LINE

The exosomes are produced from an RBL (Rat Basophilic Leukemia) line, transfected in a stable way to express, on the exosome membrane, human MHC II molecules (PCT/FR99/02691). After induction, with an ionophore (iomicyne), the cells degranulate and release exosomes in a protein-free medium (RPMI). After the cells are eliminated by centrifugation at 600 g for 10 min, the supernatants are recovered and treated with a DNase (Sigma) and an RNase (Sigma)

The treated supernatants are then centrifuged at 10,000 g at 37°C for 30 min and then at 100,000 g for 1 hour.

The ultracentrifugation sediment is loaded onto an ion exchange column, Source 15Q, Pharmacia (fig. 9). A 0.8 ml column is used with a 5 ml/min flow rate (linear flow rate 1880 cm/hour, contact time 0.1 min).

The column is equilibrated in 12 mM BTP buffer (150 mM NaCl, pH 7). After loading the sample, the column is washed with 15 to 20 column volumes of the same equilibrating buffer. The elution is performed with 25 column volumes by varying the saline concentration from 150 mM to 1 M NaCl, the pH is kept constant (fig. 9). The protein contamination of the medium is very low since the cells are washed in PBS and the induction and release of the exosomes are performed in RPMI only. This is confirmed by the absorption observed (280 and 254 nm) in the non-adsorbed fraction of Source 15Q. The presence of the exosomes is confirmed by a Western Blot analysis targeted against MHC II molecules (fig. 11)

after separation of the peaks with steps in NaCl (fig. 10) and ultracentrifugation of each of the peaks.

Three peaks plus one non-adsorbed fraction are observed: a first peak eluted at 350 mM NaCl, a second peak eluted at 700 mM NaCl and finally a third peak eluted at 1.5 M NaCl. The peak at 700 mM is the majority peak. In a Western Blot analysis targeted against human MHC II molecules, a signal is detected in peaks 1 (350 mM NaCl) and 2 (700 mM NaCl).

It should be noted that peak 1, which has a protein signal intensity 7 to 8 times lower than peak 2, shows a Western Blot signal equivalent to that of peak 2. This may convey a higher purity of peak 1. To confirm this fact, the peaks were ultracentrifuged and observed in electron microscopy.

Peak 1 is very rich in exosomes labelled with an anti-human MHC II antibody and there is little or no background noise. The exosomes are heterogeneous in size and there does not appear to be a separation of the exosomes by their size but by a specific competition mechanism between the eluant (NaCl) and the exosomes.

The fractions between the two peaks were analysed by electron microscopy. Few exosomes were detected with a higher background noise than in peak 1.

Peak 2 is very similar to the fractions between the two peaks; few exosomes and a higher background noise are detected.

Conclusions

Source 15Q is able to retain exosomes and separate them from potential contaminants (fig. 9). The vast majority of the exosomes are eluted in the same peak at 5 350 mM NaCl. In electron microscopy, the exosomes appear "normal" with a specific label for human MHC II molecules. The heterogeneity of the size of the exosomes (peak 1) appears to indicate that the separation is based on ion exchanges and not on 10 screening. Therefore, Source 15Q may be used as a purification step for RBL exosomes.

c) an anion exchange chromatography and/or gel permeation chromatography treatment of the sample.

7. Process according to claims 5 or 6, characterised in that the enrichment step comprises a clarification stage, optionally followed by a concentration stage.

8. Process according to any one of claims 5 to 7, characterised in that the enrichment step comprises an affinity chromatography step, preferably on a dye.

9. Process according to claim 7 or 8, characterised in that the enrichment step comprises a low speed centrifugation step and/or a filtration.

10. Process according to any one of claims 7 to 9, characterised in that the enrichment comprises at least an ultrafiltration step, particularly tangential.

11. A process of preparing membrane vesicles, characterised in that it comprises the following steps :

a) the culture of a population of membrane vesicle (e.g. exosome) producing cells under conditions enabling the release of vesicles,

b) the treatment of the culture supernatant with at least one ultrafiltration or affinity chromatography step, to produce a biological sample enriched with membrane vesicles (e.g. with exosomes), and

c) an anion exchange chromatography and/or gel permeation chromatography treatment step of the biological sample.

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12. Process according to claim 11, characterised in that it further comprises a filtration step d) of the treated preparation.

13. Process according to any of the above claims, characterised in that the membrane vesicles have a diameter between approximately 60 and 90 nm.

14. Process according to any of the above claims, characterised in that the membrane vesicles are vesicles produced by antigen presenting cells, particularly dendritic cells, B lymphocytes, macrophages or mastocytes.

15. Process according to claim 14, characterised in that the membrane vesicles are vesicles produced by dendritic cells, particularly of human origin.

16. Process according to any of claims 1 to 13, characterised in that the membrane vesicles are vesicles produced by tumoral cells, particularly of human origin.

17. A process of preparing membrane vesicles, characterised in that it comprises the following steps:

- a) obtaining a population of dendritic cells,
- b) culturing the dendritic cells under conditions enabling the production of membrane vesicles, and
- c) purifying the membrane vesicles using a process comprising at least an anion exchange chromatography treatment.

18. A process of preparing membrane vesicles, characterised in that it comprises the following steps:

- a) obtaining a population of dendritic cells,

b) culturing the dendritic cells under conditions enabling the production of membrane vesicles,

c) treating the culture supernatant to produce a biological sample enriched with membrane vesicles,
5 particularly by an ultrafiltration or affinity chromatography step, and

d) purifying the membrane vesicles using a process comprising at least an anion exchange and/or gel permeation chromatography step.

10 19. Process according to claim 17 or 18, characterised in that the dendritic cells are obtained from a biological sample from a subject, e.g. bone marrow or peripheral blood.

15 20. Process according to claims 17 to 19, characterised in that the dendritic cells are immature.

21. Process according to any of claims 17 to 20, characterised in that the dendritic cells are sensitised to an antigen, prior to the membrane vesicle production.

20 22. Process according to any of claims 17 to 21, characterised in that, during step b), the dendritic cells are cultured under conditions stimulating membrane vesicle production.

25 23. Use of anion exchange chromatography for the preparation or purification of membrane vesicles.

24. Use of affinity chromatography for the preparation or purification of membrane vesicles.

25 25. Composition comprising membrane vesicles prepared using the process according to any of claims 1
30 to 22.

ABSTRACTMETHODS FOR PREPARING MEMBRANE VESICLES

This invention relates to a method of preparing membrane vesicles from a biological sample, characterised in that it comprises at least one anion
5 exchange and/or gel permeation chromatography treatment of the sample. The invention is used to prepare vesicles of varied origins and types, particularly from antigen presenting cells or tumoral cells. The invention also relates to the vesicles obtained in this
10 way and their uses.

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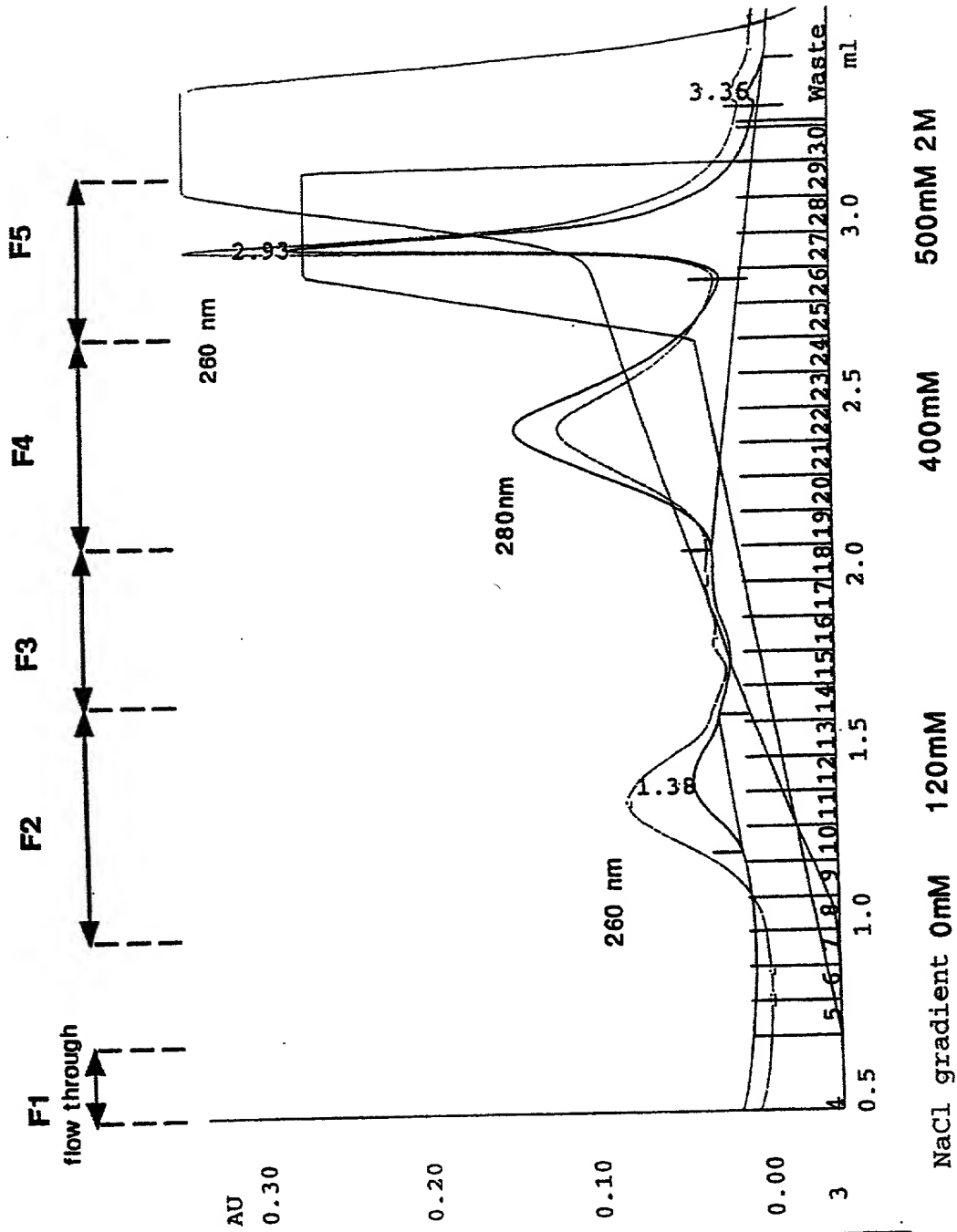


Figure 1

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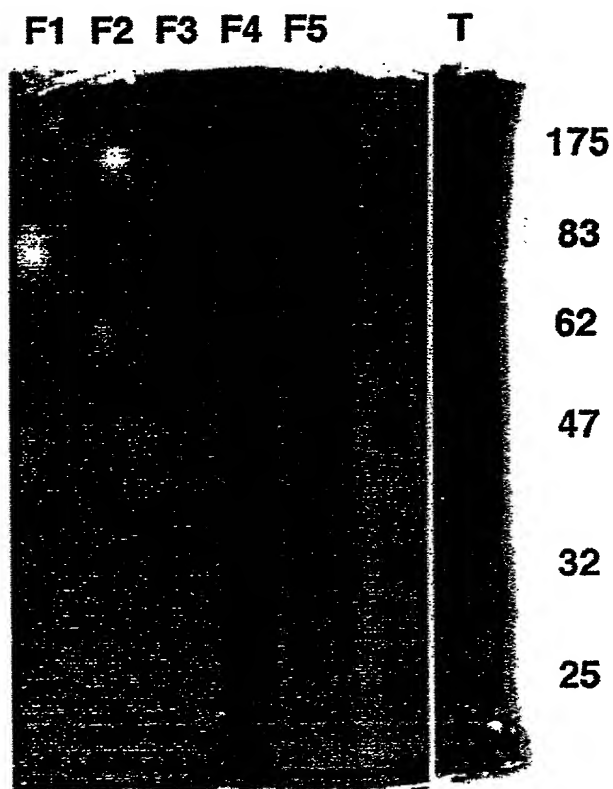


Figure 2

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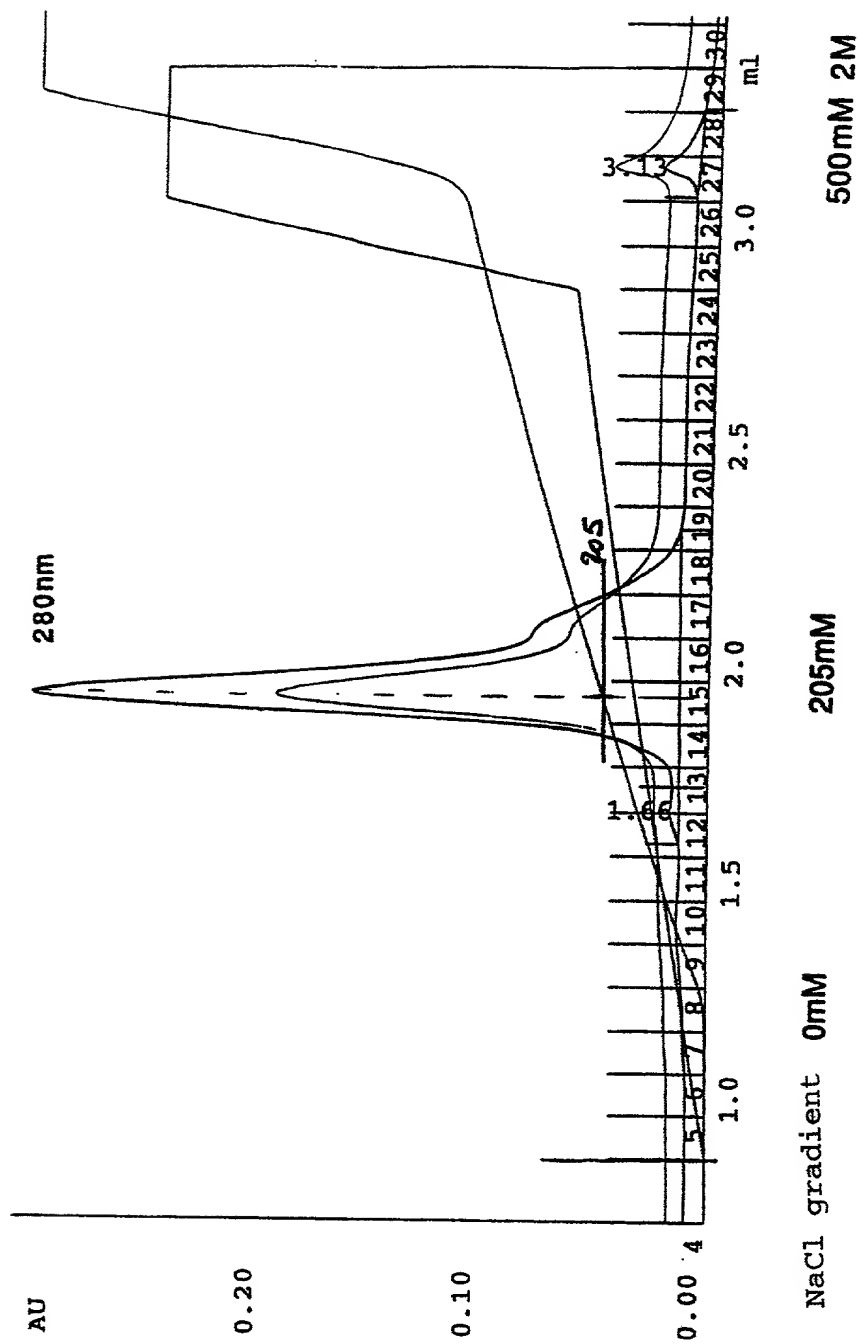


Figure 3

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Tangential filtration

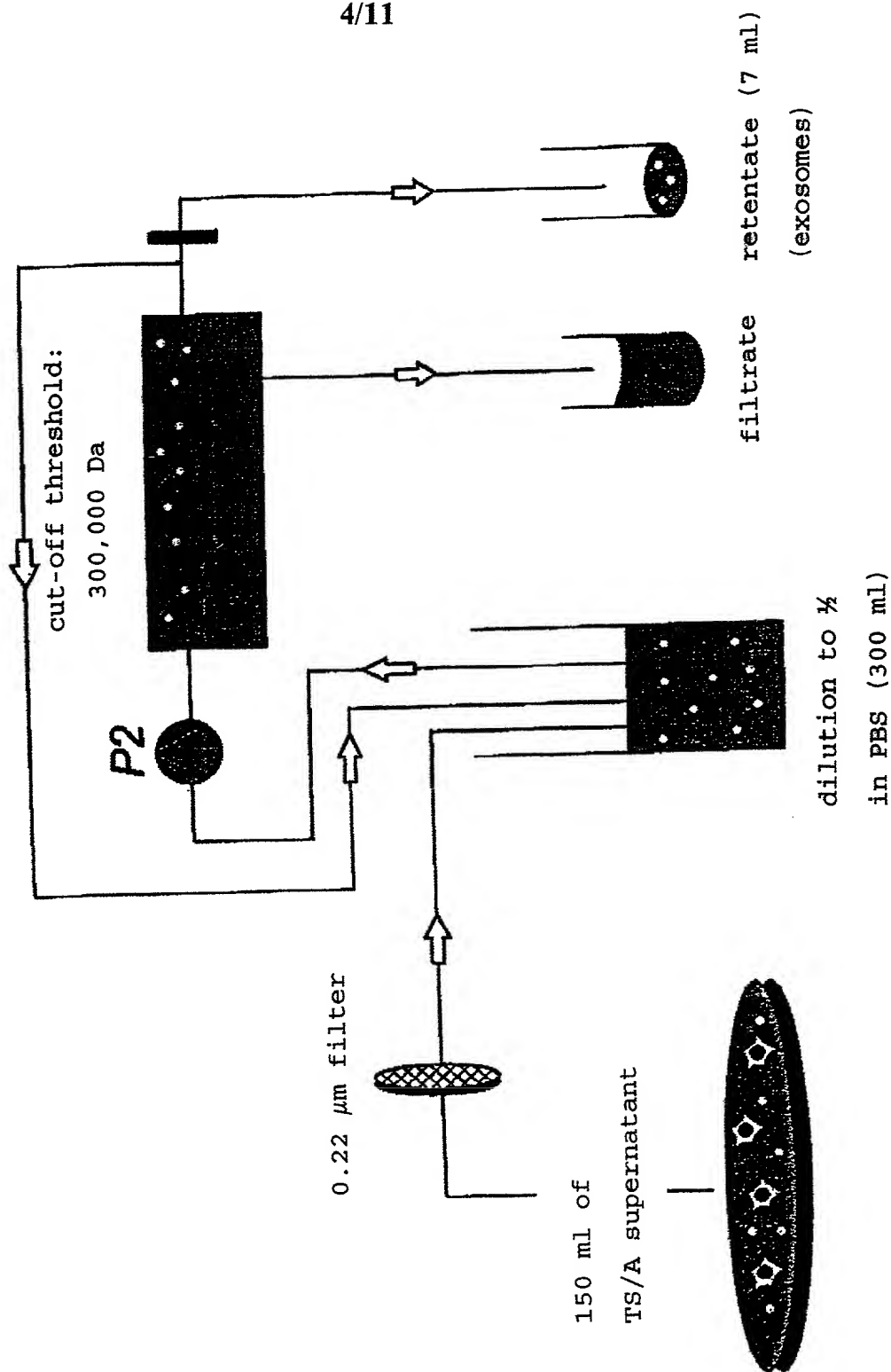


Figure 4

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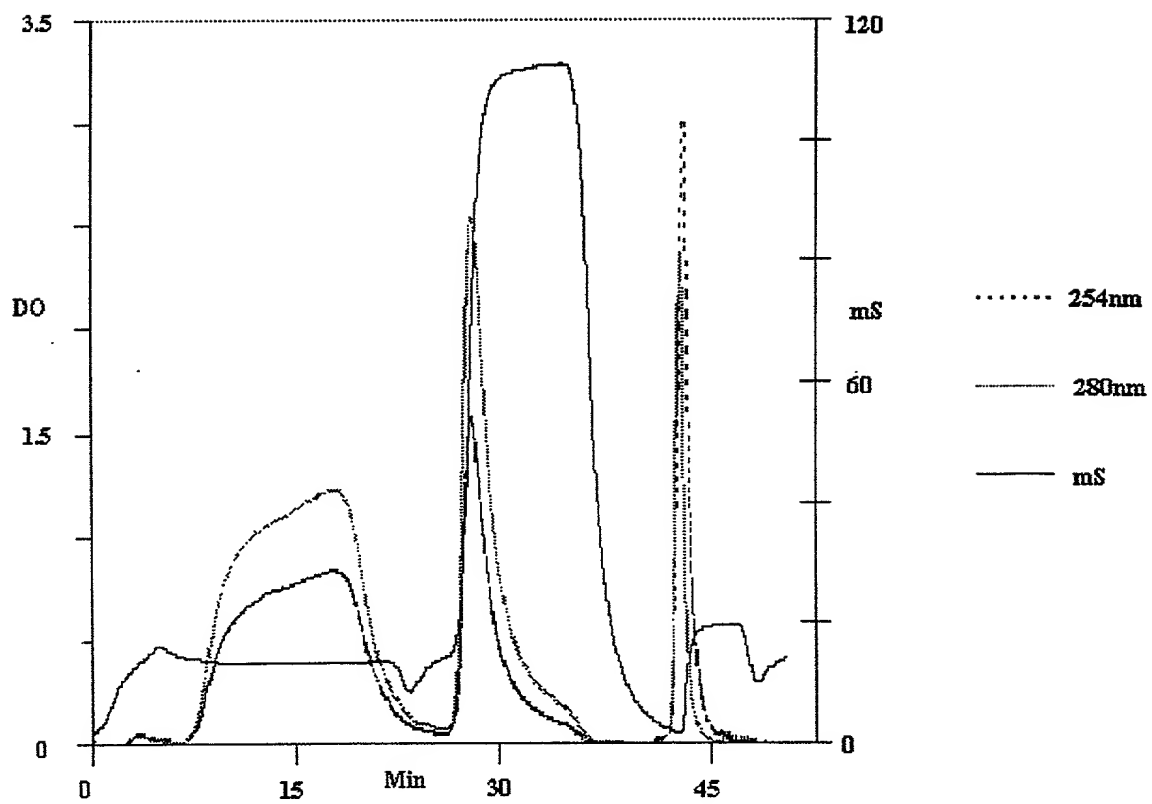


FIGURE 5

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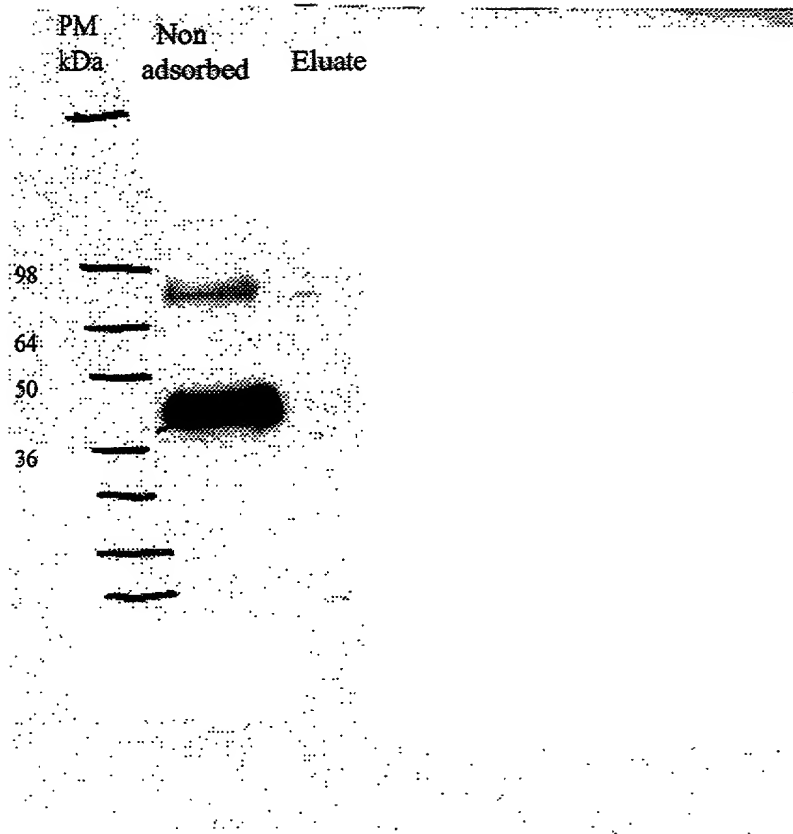


FIGURE 6

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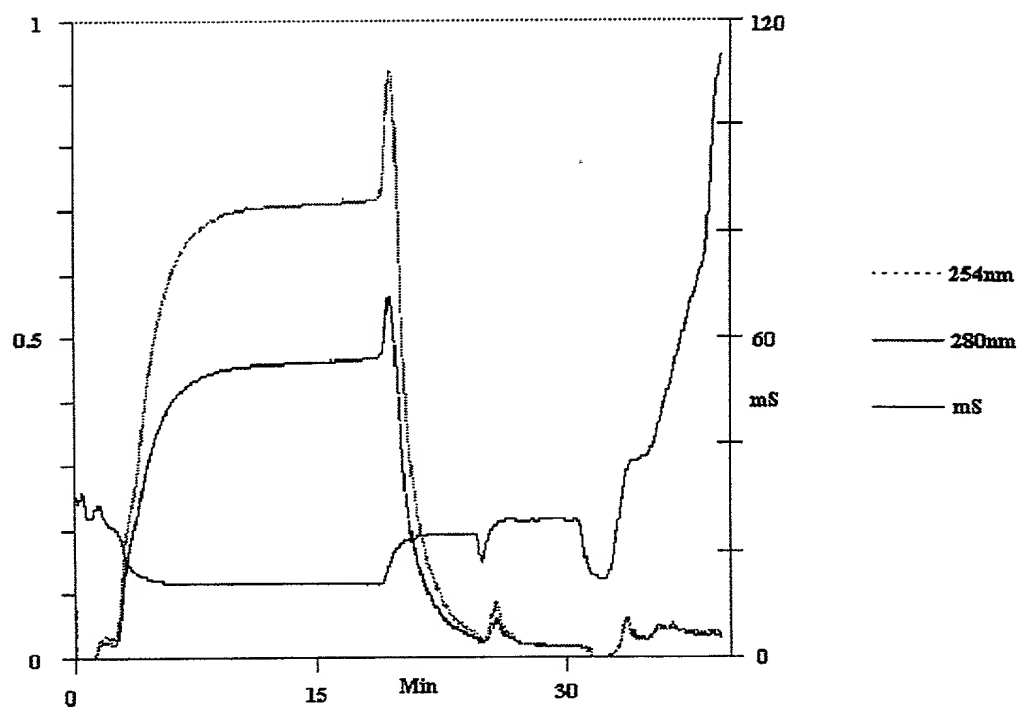


FIGURE 7

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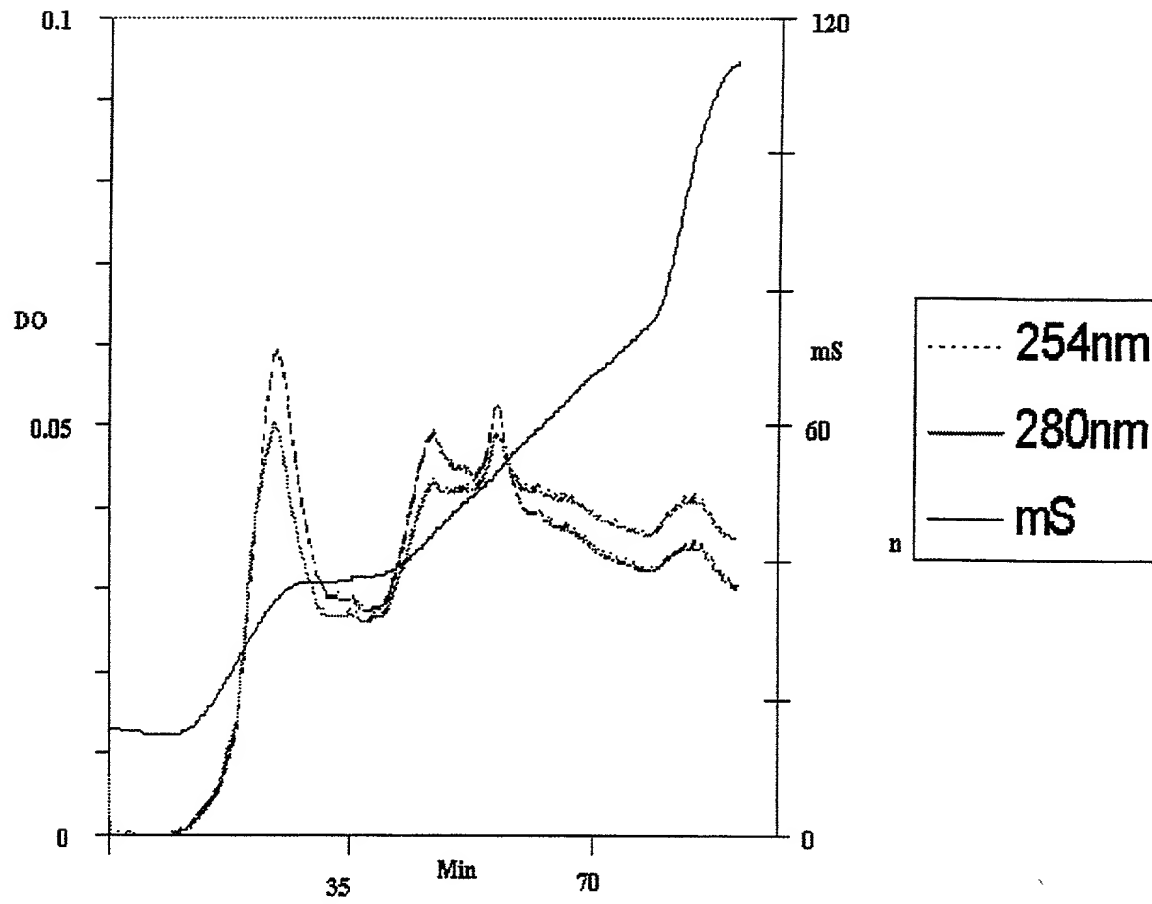


FIGURE 8

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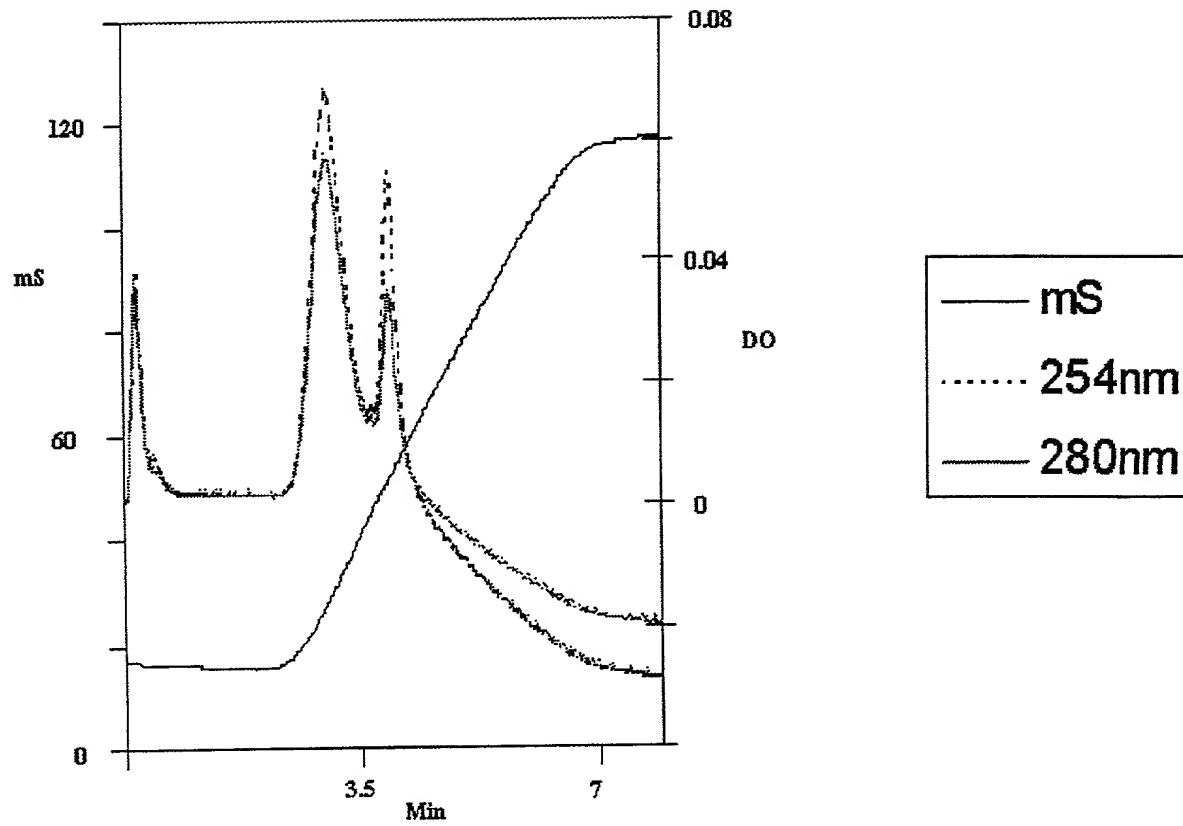


FIGURE 9

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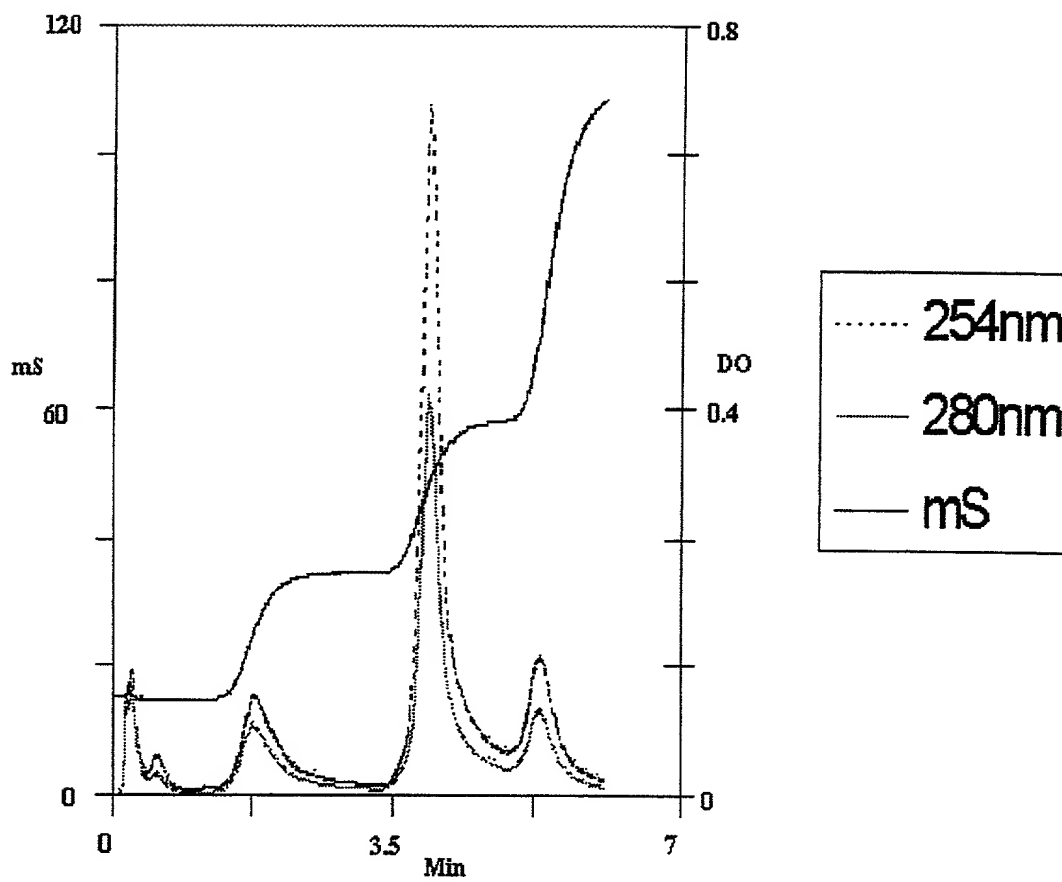


FIGURE 10

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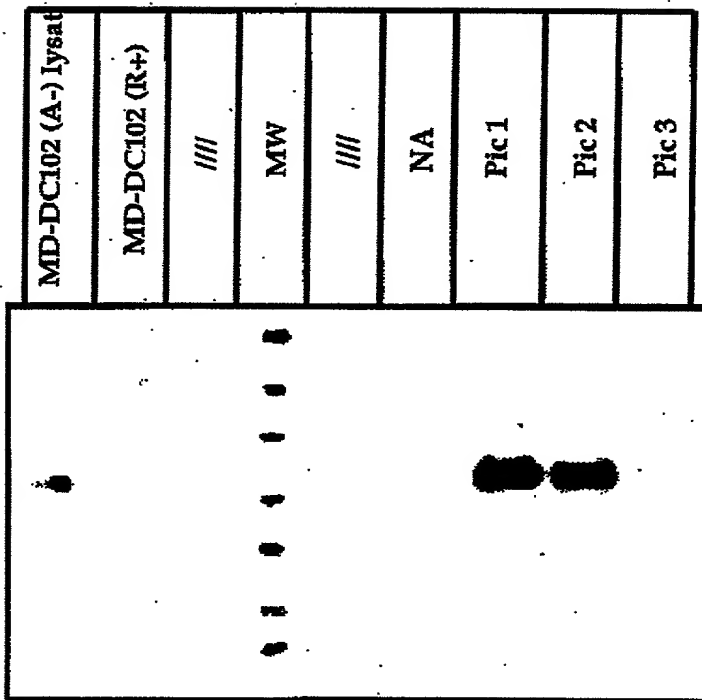


FIGURE 11

FOIA b 7 E.O. 13526

**POWER OF ATTORNEY
By Assignee**

AP CELLS, INC., assignee(s) of the application for United States Letters Patent for an improvement in

METHOD FOR PREPARING MEMBRANE VESICLES

by Olivier DHELLIN, Sebastian AMIGORENA, Philippe RAMEAU, and Joel CROUZET

the specification of which:

- ☒ is filed herewith, OR
☐ was filed on _____, having U.S. Patent Application Serial No. _____,

does hereby appoint as my attorneys and/or agents, with full power of substitution and revocation, to prosecute this application and transact all business in the United States Patent and Trademark Office, and in countries other than the United States, and to do all things necessary or appropriate therefor before any competent International Authorities in connection with any international patent application(s) corresponding to the above-identified application, all of the registered practitioners identified by Customer Number 22249:

**22249**

PATENT TRADEMARK OFFICE

LYON & LYON LLP
 Suite 4700
 633 W. Fifth Street
 Los Angeles, CA 90071
 (213) 489-1600

Please send all inquiries to Kurt T. Mulville, at the above Customer Number.

I, the undersigned, declare that I have reviewed copies of the documentary evidence establishing chain of title to the patent application identified above from the inventor(s) to the assignee(s), which:

- ☒ is filed for recordation herewith; or
☐ was recorded at Reel _____, Frame _____; or
☐ has been sent for recordation under separate cover, copy attached herewith.

To the best of the undersigned's knowledge and belief, title is in the assignee(s) identified above. Furthermore, the undersigned is empowered to sign this document on behalf of the assignee(s).

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09220 6120860

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	FULL NAME OF INVENTOR	FIRST Name	MIDDLE Initial	LAST Name	

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Date	
Signature of Inventor	202
Date	
Signature of Inventor	203
Date	

Signature of Inventor	204
Date	16.06.2001
Signature of Inventor	
Date	

(Signatures should conform to names as presented at 201 et seq. above.)

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Signature of Inventor	202
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Signature of Inventor	203
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Signature of Inventor	204
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(Signatures should conform to names as presented at 201 et seq. above.)

FOSSCO 676666

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Signature of Inventor	203
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Signature of Inventor	204
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Signature of Inventor	
Date	

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**DECLARATION
Utility Application**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **METHOD FOR PREPARING MEMBRANE VESICLES** the specification of which

(Check One) ☒ is attached hereto OR
☐ was filed on _____ as United States Application Serial No. _____ or
PCT International Application No. _____ and was amended on _____ (if
applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Date of Filing	Priority Claimed	
			Yes	No
Serial No. 99 00886	France	January 27, 1999	X	

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date

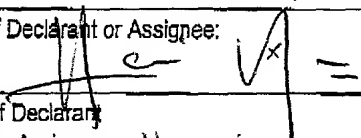

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date	Status-Patented, Pending or Abandoned

Send Correspondence to: <u>Kurt T. Mulville</u>	<u>LYON & LYON LLP</u> <u>633 W Fifth St., Suite 4700</u> <u>Los Angeles, CA 90071</u>	Direct Telephone calls to: 949/567-2300 X 1124
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Patent
265/130

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Title of Declarant:	
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